Austin Journal of Anatomy

Open Access

Austin Publishing Group

Research Article

Molecular Identification of *Cryptosporidium* spp. and *Giardia* spp. in Wild Birds in Southeastern Brazil

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Received: March 02, 2021; **Accepted:** April 09, 2021; **Published:** April 16, 2021

Abstract

Introduction: The identification of *Cryptosporidium* spp., *Giardia* spp. and *Enterocytozoon bieneusi* in birds is relevant since these animals can act as disseminators of these parasites to humans through environmental contamination. The aim of this study was to determine the molecular occurrence of *Cryptosporidium* spp. and *Giardia* spp. in wild birds in southeastern Brazil and genetically characterize the isolates obtained.

Methods: A total of 256 fecal samples were collected from 172 captive and 84 free-living wild birds. The DNA extracted was subjected to nested-PCR and semi-nested PCR analysis for amplification of fragments of the 18S rDNA and *gdh* genes of *Cryptosporidium* spp. and *Giardia* spp., respectively.

Results: With respect to *Cryptosporidium* spp., the overall occurrence was 3.91%. Of samples from captive wild birds, six (3.49%) were positive: two waterfowl and four non-aquatic birds. Among the samples from free-living wild birds, four (4.76%) were positive, all non-aquatic birds. Regarding *Giardia* spp., the overall occurrence was 3.1%. Of samples from captive wild birds, four (2.32%) were positive, all waterfowl; of the samples from free-living wild birds, four (4.76%) were positive for the parasite, all non-aquatic birds.

Conclusions: The presence of *C. meleagridis* and *G. duodenalis* assemblage B suggests that epidemiological studies involving wild birds and humans are needed to better understand the impact of avian cryptosporidiosis and giardiasis on avian health and their possible implications for public health.

Keywords: *Cryptosporidium* spp.; *Giardia* spp.; Wild birds; 18S rDNA; *gdh*

Introduction

Cryptosporidiosis and *Giardia*sis are zoonotic gastrointestinal diseases in immunocompetent and immunocompromised worldwide [1]. Besides humans, *Cryptosporidium* spp. and *Giardia* spp. infect a wide range of vertebrate hosts including domestic and wild birds [2].

Cryptosporidiosis is one of the most prevalent parasitic infections in birds and has been found in more than 30 avian species from all continents, except Antarctica [2]. So far, four species of *Cryptosporidium* are recognized in birds: *C. meleagridis*, *C. baileyi*, *C. galli* and *C. avian*. They differ from each other in their host range, infection sites, and symptomatology associated with infection. In addition, several genotypes have been described in birds worldwide, including avian genotypes I-VI, goose genotypes I-V, black duck genotype, and Eurasian woodcock genotype [3]. Among them, only *C. meleagridis* is known to also infect mammals [4] and has public health significance since it is the third most common cause of cryptosporidiosis in humans [2,5].

Giardia spp. is commonly found infecting the intestine of several avian hosts. Two *Giardia* species are responsible for giardiasis in birds, *G. psittaci* and *G. ardeae* [6]. In addition to these two species, the zoonotic assemblages A and B as well as non-zoonotic assemblages D and F of *G. duodenalis* have been found in birds (Reboredo-Fernández et al. 2015; Majewska et al. 2009) implying that these animals may be directly involved in maintaining the transmission cycles of zoonoses [3].

Although previously studies have indicated that poultry could play an important role in the transmission of zoonotic parasites for humans and other animals, the role of wild birds in the dissemination of *Cryptosporidium* spp. oocyst and *Giardia* spp. cysts is still unclear. The aim of this study was to investigate the molecular occurrence of *Cryptosporidium* spp. and *Giardia* spp. in wild birds from Triangulo Mineiro, Brazil and genetically characterize the isolates obtained.

Material and Methods

From March 2013 to February 2014, 218 fecal samples were obtained from captive and free-living wild birds at the ambulatory of the Research Laboratory in Wild Animals (LAPAS) of the Federal University of Uberlandia (UFU). The ambulatory provides medical assistance for wild animals from the microregion of Uberlandia brought by environmental agencies and population. The birds comprised 29 species belonging to 16 families (Table 1). In addition, 38 samples from waterfowl (Family Anatidae) at the Municipal Zoological Park of Sabia in Uberlândia, Minas Gerais, Brazil, were included in the study (Table 1). All birds at the zoo were considered captive animals. Of the 256 wild birds, 172 (67.2%) were captive and 84 (32.8%) were free-living; 39 (15.2%) were waterfowl, and 217 (84.8%) were non-aquatic birds.

Citation: da Cunha MJR, Santos ALQ, Silva MBO, dos Santos MC, Fava NMN and Figire Cury MC. Molecular Identification of *Cryptosporidium* spp. and *Giardia* spp. in Wild Birds in Southeastern Brazil. Austin J Anat. 2021; 8(1): 1097.

To collect the samples all the animals were placed in individual sanitized cages and fresh feces were collected from the bottom of the cages. Feces were stored in labeled polystyrene tubes, transferred to the Laboratory of Parasitology of UFU and held at -20ºC until DNA extraction.

DNA was extracted from feces using the QIAamp Stool Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instruction with minor modifications. It was added 0.3 g of zirconia beads to 0.2 g of feces and 1.4 ml lysis buffer [7]. Then, the mixture was heated at 95ºC for 5 min followed by vigorous shaking (two rounds of 15 min) to facilitate the parasite rupture.

Nested-PCR (nPCR) was conducted to amplify 819-825 bp fragments of 18S rDNA gene of *Cryptosporidium* spp. [8,9]. Furthermore, the isolates were classified into species by PCR-Restriction Fragment Length Polymorphism (PCR-RFLP), using *SspI* and *VspI* endonuclease, as previously described (referencia). Semi-nested PCR (snPCR) was performed to amplify a 432 bp fragment of *Giardia gdh* gene according to Read et al. [10].

The nPCR and snPCR products were purified and sequenced in both directions, using the same PCR primers used in the secondary PCR using BigDye 3.1v Chemistries and an ABI 3130 sequencer analyzer (Applied Biosystems, Foster City, California). Nucleotide sequences obtained in this study were aligned, examined, and compared with reference sequences downloaded from GenBank using SeqMan™ (DNAstar Inc., Madison, Wisconsin). The nucleotide sequences obtained in this study have been deposited in GenBank under accession numbers KJ787011 to KJ787014.

Results

Data were compiled and analyzed with BioEstat 5.0 [11]. To compare two independent samples, the binomial test of two proportions was used. Statistical significance was defined as $P<0.05$.

Positive amplification for *Cryptosporidium* spp. was obtained in 10 (3.91%) samples by nPCR (Table 2). Of 172 captive birds, six (3.49%) were positive for the parasite: two muscovy duck, three blue-fronted parrots and one orange-winged parrot. Four (4.76%) of 84 free-living birds were *Cryptosporidium* spp. positive: two white-eyed parakeets and two burrowing owls (Table 2). There were no significant differences between the occurrence of *Cryptosporidium* spp. in captive and free-living birds (P=0.62) or between aquatic and non-aquatic birds (P=0.67).

RFLP analysis of the 18S rDNA gene products showed the presence of two species in positive samples, *C. meleagridis* and *C. baileyi*. *C. meleagridis* was identified in two captive muscovy duck, two free-living white-eyed parakeets, and two free-living burrowing owls. *C. baileyi* was observed in three blue-fronted parrots and one orange-winged parrot, all captive birds (Table 3).

The sequences from muscovy duck, white-eyed parakeets, and burrowing owls were identical, and when submitted to BLAST showed 100% similarity to *C. meleagridis* (JX416368.1). The sequences from the blue-fronted parrot were identical and 100% similar to *C. baileyi* (JQ413445.1), similarly the isolate from the orange-winged parrots was identical to the sequence GQ426096.1 of *C. baileyi* (Table 3).

Among the 256 samples collected, 8 (3.12%) were positive for *Giardia* spp. by the snPCR (Table 4). Of 172 captive birds, 4 (2.32%) muscovy duck were positive for the parasite, and 4 (4.76%) were positive among the 84 free-living birds: one striped owl, one buff-necked ibis and two roadside hawks (Table 4).

There was no significant difference between captive and free-living birds in the occurrence of *Giardia* spp. (P=0.29), but the occurrence

Table 1: Species, family, common names and number of birds examined for *Cryptosporidium* and *Giardia* species in Brazil.

Species	Family	Common name	n
Brotogeris chiriri [a,b,2]	Psittacidae	Yellow-chevroned parakeet	30
Aratinga leucophthalma [a,b,2]	Psittacidae	White-eyed parakeet	32
Amazona aestiva [a, b, 2]	Psittacidae	Blue-fronted parrot	56
Amazona Amazonian [b, 2]	Psittacidae	Orange-winged parrot	4
Amazona xanthops [b,2]	Psittacidae	Yellow-faced parrot	7
Aratinga aurea [a,b,2]	Psittacidae	Peach-fronted parakeet	6
Melopsittacus undulatus [b,2]	Psittacidae	Budgerigar	1
Diopsittaca nobilis [b,2]	Psittacidae	Red-shouldered macaw	2
Ara ararauna [a,2]	Psittacidae	Blue-and-yellow macaw	2
Pitangus sulphuratus [a,2]	Tyrannidae	Great Kiskadee	8
Mimus saturninus [a,2]	Mimidae	Chalk-browed Mockingbird	2
Rupornis magnirostris [a,2]	Accipitridae	Roadside hawk	10
Heterospizias meridionalis [a,2]	Accipitridae	Savanna hawk	3
Polyborus plancus [a,2]	Falconidae	Southern Caracara	11
Falco sparverius [a,2]	Falconidae	American kestrel	2
Coragyps atratus [a,2]	Cathartidae	Black vulture	3
Asio clamator [a,2]	Strigidae	Striped owl	1
Athene cunicularia [a,2]	Strigidae	Burrowing owl	6
Tyto alba [a,2]	Tytonidae	Barn owl	2
Ramphastos toco [a,b,2]	Ramphastidae	Toco Toucan	10
Eupetomena macroura [a,2]	Trochilidae	Swallow-tailed hummingbird	2
Colibri serrirostris [a,2]	Trochilidae	White-vented Violetear	1
Columbina talpacoti [a,2]	Columbidae	Ruddy Ground-dove	1
Columba livia [a,2]	Columbidae	Rock pigeon	7
Nymphicus hollandicus [b,2]	Cacatuidae	Cockatiel	3
Gnorimopsar chopi [a,2]	Icteridae	Chopi blackbird	1
Sporophila angolensis [a,2]	Emberizidae	Chestnut-bellied Seed-Finch	2
Gallinula galeata [a,1]	Rallidae	Common Gallinule	2
Theristicus caudatus [a,2]	Threskiornithidae	Buff-necked Ibis	1
Cygnus atratus [b,1]	Anatidae	Black Swan	2
Chloephaga rubidiceps [b,1]	Anatidae	Ruddy-headed Goose	9
Alopochem aegyptiacus [b,1]	Anatidae	Egyptian Goose	7
Cairina moschata [b,1]	Anatidae	Muscovy duck	20
Total			256

[a]Free-living birds.
[b]Captive birds.
[1]waterfowl.
[2]Non-aquatic birds.

Table 2: Occurrence of *Cryptosporidium* species in captive and free-living wild birds in Brazil.

Avian host	Captive	Positive	Free-living	Positive	Sample size
	n	n	n	n	
White-eyed parakeet[2]	26	0	6	2	32
Blue-fronted parrot[2]	55	3	1	0	56
Orange-winged parrot[2]	4	1	0	0	4
Burrowing owl[2]	0	0	6	2	6
Muscovy duck[1]	38	2	0	0	38
25 other species[1,2]	49	0	71	0	120
Total	172	6 (3.49%)	84	4 (4.76%)	256 (3.91%)

[1]Waterfowl.
[2]Non-aquatic birds.

Table 3: Isolate of avian species of *Cryptosporidium* spp., the hosts in which they were found and results of PCR-RFLP and sequencing.

Avian host	*Enzyme *SspI*	*Enzyme *VspI*	RFLP	Blast	Similarity
	(pb)	(pb)	result	result	
Muscovy duck	108, 254, 449	102(104), 171, 456	*C. meleagridis*	*C. meleagridis*	100%
Muscovy duck	108, 254, 449	102(104), 171, 456	*C. meleagridis*	*C. meleagridis*	100%
Blue-fronted parrot	254, 572	102(104), 620	*C. baileyi*	*C. baileyi*	100%
Blue-fronted parrot	254, 572	102(104), 620	*C. baileyi*	*C. baileyi*	100%
Blue-fronted parrot	254, 572	102(104), 620	*C. baileyi*	*C. baileyi*	100%
Orange-winged parrot	254, 572	102(104), 620	*C. baileyi*	*C. baileyi*	100%
White-eyed parakeet	108, 254, 449	102(104), 171, 456	*C. meleagridis*	*C. meleagridis*	100%
White-eyed parakeet	108, 254, 449	102(104), 171, 456	*C. meleagridis*	*C. meleagridis*	100%
Burrowing owl	108, 254, 449	102(104), 171, 456	*C. meleagridis*	*C. meleagridis*	100%
Burrowing owl	108, 254, 449	102(104), 171, 456	*C. meleagridis*	*C. meleagridis*	100%

*Xiao *et al.* (1999); Xiao *et al.* (2001).

Table 4: Occurrence of *Giardia* species in captive and free-living wild birds in Brazil.

Avian Host	Captive	Positive	Free-living	Positive	Sample size
	n	n	n	n	
Striped owl[2]	0	0	1	1	1
Buff-necked Ibis[2]	0	0	1	1	1
Roadside hawk[2]	0	0	10	2	10
Muscovy duck[1]	38	4	0	0	38
26 other species[1,2]	134	0	72	0	206
Total	172	4 (2.32%)	84	4 (4.76%)	256 (3.12%)

[1]Waterfowl.
[2]Non-aquatic birds.

of the parasite was significantly higher in waterfowl (P=0.0054).

Two snPCR-positive samples from the roadside hawk were sequenced. The isolates were identical and a BLAST search showed 100% similarity to *G. duodenalis* assemblage B (GenBank Accession number JN204452.1).

Discussion

This study demonstrated the presence of *Cryptosporidium* and *Giardia* in wild birds from southeastern Brazil. This is the first identification of *Cryptosporidium* in burrowing owl and *Giardia* in striped owl, buff-necked Ibis, roadside hawk and muscovy duck. *Cryptosporidium* is a relevant pathogen found in birds worldwide [3]. In Brazil, it has been previously reported in domestic, wild, exotic, and captive birds [3,12,13]. In this study, *Cryptosporidium* was detected in 3.91% of fecal samples examined. Similar prevalence was reported in captive birds [3], however it was lower than those found in wild, captive, exotic and domestic birds in Brazil [3,12,13].

For *Giardia* spp. the occurrence was slightly lower than described by Plutzer et al. [14], which reported 5 to 49% prevalence. This difference in results might be attributed to the different diagnostic techniques used [15].

No significant differences were observed between captive and free-living birds in the detection of *Cryptosporidium* spp. and *Giardia* spp. This differed from Majewska et al. who reported higher prevalence in free-living birds. Free-living birds are presumed to

be more susceptible to pathogens, since they are in contact with varying environmental conditions, in contrast to birds in captivity, where controlled conditions might prevent exposure to parasites. The difference in results may be attributed to factors such as sample size, bird management, method of diagnosis, and geographic location.

The capacity of waterfowl for delivering human pathogens to surface water has been described for several authors

No significant difference was found between waterfowl and non-aquatic birds in the occurrence of *Cryptosporidium* spp., while positivity for *Giardia* spp. was significantly higher in waterfowl. Majewska et al. found higher infection rates by both parasites in waterfowl compared to non-aquatic birds. The finding of oocysts and cysts of zoonotic protozoa in aquatic birds suggests a risk to public health, since these pathogens are a source of disease associated with drinking and recreational waters [16].

Among the two *Cryptosporidium* species identified in this study, *C. meleagridis* predominated, unlike previous studies reporting *C. bailey* to be the most common avian *Cryptosporidium* species [2,16,17].

C. meleagridis appears to have a wide range of avian hosts including chickens, turkeys, parrots, cockatiels, red-legged partridge, and rose-ringed parakeets [18-26]. *C. meleagridis* has been identified essentially in birds but also in humans and many other mammals [5,27-29]. According to Ryan [2], *C. meleagridis* is an emerging human pathogen and is the third most common *Cryptosporidium* parasite in humans [4].

The identification of *C. meleagridis* in free-living birds in this study suggests risk of environmental dissemination. In view of its status as an emerging human pathogen, its presence in captive muscovy duck from zoo may have implications for public health, as some animals move freely through the site, where they are in contact with handlers and visitors.

C. baileyi have greater specificity for avian hosts and are often associated with respiratory cryptosporidiosis, with high morbidity and mortality in birds, especially in broilers [20]. In this study, the presence of *C. baileyi* in birds of genus Amazona, which are native to South America, was observed. Recently, in Brazil, *C. baileyi* has been identified in the black vulture, saffron finch, buffy-fronted seedeater, goldfinch, and red-cowled cardinal [3,11].

Giardia spp. have been identified in birds including Psitaciformes, Anseriformes, Gruiformes, Ciconiiformes, and Passeriformes [30-32]. To the best of our knowledge, this is the first report of the parasite in striped owl, buff-necked Ibis, roadside hawk and muscovy duck.

The *gdh* gene sequencing of *Giardia* spp. failed in some samples positive by snPCR. According to Nakamura et al. [3], losses or poor quality of the amplified DNA, a small number of cysts in the samples, and a small amount of amplified DNA may be responsible for these failures.

The sequencing identified *G. duodenalis* in the samples evaluated. According to Feng and Xiao [33], *G. duodenalis* is a multispecies complex, due to the presence of various genotypes and subgenotypes, which may be zoonotic or host-specific. Among the genotypes of *G. duodenalis*, assemblages A and B have the broadest host specificity and have been found to infect humans and other vertebrates, including birds [34]. In this study, the samples sequenced were characterized as assemblage B. Plutzer and Tomor [31], working with waterfowl, have found most animals infected with genotype B. Nevertheless, Feng and Xiao [33] commented that genotype A is more prevalent than genotype B in wild animals.

The identification of *G. duodenalis* zoonotic genotypes in wild birds highlights the potential role of these animals in the maintenance of the zoonotic transmission cycle of giardiasis. According to Karanis et al. [15] and Baldursson and Karanis [35-45], the primary protozoa involved in outbreaks of waterborne diseases are *Giardia* spp. and *Cryptosporidium* spp.

Although *Giardia* species have not been characterized in zoo waterfowl, the potential risks to humans and animals posed by the presence of this parasite in birds should be considered. If the genotype was zoonotic, handlers and visitors could be exposed to infection due to direct contact with the animals, but if the genotype identified was specific, the animals could become reservoirs of infection for uninfected birds.

Conclusion

Although studies have demonstrated the role of birds in habitat contamination by human pathogens, little is known about the mechanisms and factors associated with host and parasite that facilitate or impede the environmental contamination. Further epidemiological studies to better understand the importance of birds in dissemination of zoonotic species/genotypes of *Cryptosporidium* and *Giardia* are necessary. It is important to understand the impact of these birds on public health, especially when they are present in recreation areas such as parks and zoos, as well as near sources of drinking water.

Acknowledgment

The authors thank the staff of LAPAS and Zoological of Municipal Park of Sabia who authorized the sample collection.

The study was approved by the Ethics Committee in Animal Research of Federal University of Uberlandia (CEUA-UFU) under protocol 111/11.

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